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Award Number: W81XWH-06-1-0292

TITLE: Role of Obesity in Prostate Cancer Development

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REPORT DATE: March 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2007	Annual	01 Feb 06 – 31 Feb 07

4. TITLE AND SUBTITLE
Role of Obesity in Prostate Cancer Development

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0292

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Minnesota
Minneapolis MN 55455-2070

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Prospective epidemiological studies indicate that obesity increases the risk for prostate cancer. Also, mortality from prostate cancer is increased with elevated body weights and obesity recently was reported to be associated with higher prostate cancer grade at diagnosis and with higher recurrence rates. However, it is difficult in human studies to adequately assess effects of body weight or the effect of body weight change at specific ages on prostate cancer. Recent introduction of the TRAMP (transgenic adenocarcinoma mouse prostate) mouse provides a model that shares many characteristics with human prostate cancer. In the present study we assessing the effect of obesity induced at different ages on the development of prostate cancer in TRAMP mice. Obesity is induced in TRAMP mice by injections with gold-thioglucose (GTG) at either 6, 16 or 26 weeks of age. Body weight is monitored and longitudinal blood samples are obtained to monitor serum leptin levels. Mice are followed until 46 weeks of age. The 26-week cohort study is complete and preliminary analyses of the data indicate no effect of body weight on prostate tumor development although pathology results have not been obtained. Mice in the 6- and 16-week cohorts are currently being followed.

15. SUBJECT TERMS
TRAMP mice, obesity, prostate cancer

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	12	

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Prescribed by ANSI Std. Z39.18

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INTRODUCTION:

A number of epidemiological studies indicate that increased body weight plays a role in the development of prostate cancer [1-9]. Although, not all studies have found obesity to be associated with increased risk of prostate cancer, Bergström *et al* concluded that based on the obtained relative risk values that 5,000 new cases of prostate cancer per year in Europe could be attributed to obesity [10]. In addition, mortality from prostate cancer is increased with elevated body weights [11], and obesity was recently reported to be associated with higher prostate cancer grade at diagnosis, as well as with higher recurrence rates [12]. The potential role of body weight in the development and progression of prostate cancer is of interest given that the incidence of overweight/obesity is increasing throughout the world, and the potential for lifestyle changes to alter body weight status. Interesting since we originally submitted this proposal an increasing number of publications have addressed the issue of obesity and its association with prostate cancer prognosis. In the present study we are using the TRAMP mouse model of prostate cancer to study the effects of obesity initiated at specific ages on prostate cancer development in relation to stage of the disease process. Since prostate cancer in TRAMP mice progresses from androgen-dependent to androgen-independent status over time this will provide a realistic comparison to various stages of the human disease process. Obesity will be induced in mice at different ages (6, 16 and 26 weeks of age) using gold-thioglucose (GTG) injections.

BODY:

Although we previously used diet-induced obesity to determine effects of body weight on the development of mammary tumors, due to the variable response of mice to this intervention, *i.e*., a range of body weights obtained, we are using an alternative approach to increase body weight in this study. Our rationale is that due to the shorter time for development of prostate cancer in TRAMP mice; and the transition of the disease through different stages, a more uniformly developing obesity model will make it easier to interpret results and fewer experimental animals will be needed. An alternative to diet-induced obesity is to damage the hypothalamus [13]. This can be done by physical/mechanical means (knife cuts and electrical lesions) or less invasively by chemical damage. Specifically, injection of mice with gold-thioglucose (GTG) results in the majority of the mice gaining weight and becoming obese [14]. It is not clear why not all animals respond, because when they are re-injected they then develop obesity. This is an important observation as it indicates that the initial lack of response is not due to resistance to GTG. Shortly after leptin was identified, GTG-induced obesity was reported to increase plasma leptin as identified by immunoblot; and leptin mRNA expression in adipose tissue was elevated compared to lean animals [15]. More recently when leptin levels were assessed by commercially available radioimmunoassay kits, GTG obese mice were found to have serum leptin levels two-fold higher than in control mice [16;17]. Although food intake is initially increased in GTG-treated mice, body weight gain without consumption of a high-fat diet is obtained [16;18]. Also body weights eventually plateau and caloric intakes are appropriate for body weights. There is no age-sensitive time-point at which GTG needs to be administered in order to produce the effect on body weight. For example, in some studies mice were as young as 3-4 wks of age [19-22], while in others they were 8-12 [16;18;23;24] or 20 weeks of age [14;16;23;24].

In the literature different GTG doses over a range from 0.3-2.0 mg/kg body weight have been used. However, based on data from study in female nude mice with a dose of 0.5 mg/kg which resulted in a high mortality rate we decided to undertake a preliminary study in male mice prior to injecting the TRAMP mice with GTG.

General Methods:

All mice have ad libitum access to purified AIN-93M diet and water. Following GTG or PBS injections, mice are fasted for 24-hours and given free access to 2% glucose-supplemented water for one week Body weights are taken weekly and at that time mice are palpated for tumors. Mice who received GTG are

categorized as obese or non-obese, based on weight gain relative to the PBS control mice. Serum samples are collected from the retro-orbital sinus at baseline and every 5 weeks until a tumor is palpated. Following tumor palpation, serum is collected every 3 weeks until study termination. Data are presented as mean ± SE.

Pilot study 1

Gold thioglucose (GTG) was injected into 14 male wild-type mice at a dose of 0.5 g/kg body weight. A control group was made up of six male wild-type mice that were injected with the same dose of PBS. One mouse became ill after receiving GTG and euthanization was necessary. The mice, ranging in age from 8 to 23 weeks, were then followed for ten weeks to monitor body weight changes and general body condition. Fifty-four percent (7 out of 13) became obese. As shown in Figure 1, the GTG obese cohort gained significantly more weight than either the GTG non-obese or the control mice. There was no significant difference in the 10-week weight gain between the non-obese and the PBS injected mice.

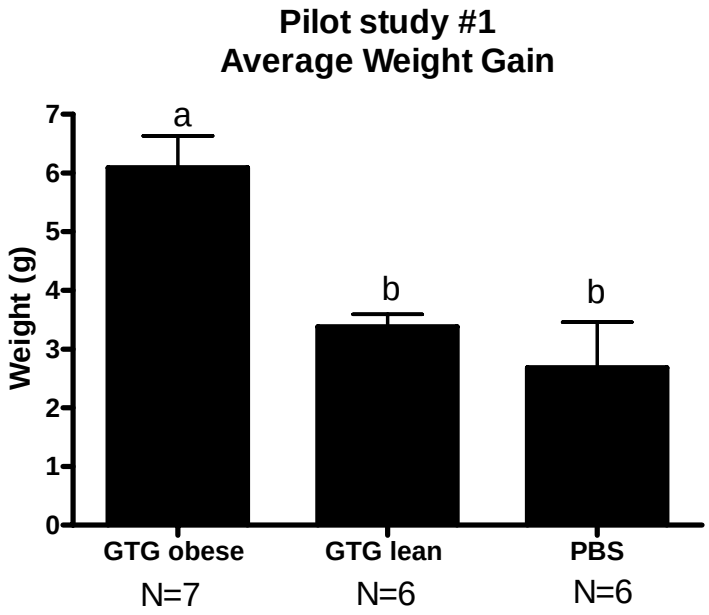

Figure 1: 10-week weight gain during pilot study 1. ANOVA P = 0.001; GTG obese versus GTG lean P < 0.01; GTG obese versus PBS P < 0.01; GTG lean versus PBS P > 0.05.

Pilot study 2

In an effort to increase the percentage of mice that became obese with GTG treatment, a dose of 0.8 g/kg body weight was injected into 12 male wild-type mice. A control group was made up of five male wild-type mice that were injected with PBS. Four mice became ill after receiving GTG and euthanization was necessary. The mice, ranging in age from 11 to 13 weeks, were then followed for ten weeks to monitor body weight changes and general body condition. Sixty-three percent (5 out of 8) became obese. As shown in Figure 2, the GTG obese cohort gained significantly more weight than either the GTG non-obese or the PBS-injected mice. There was no significant difference in the 10-week weight gain between the non-obese and the control mice.

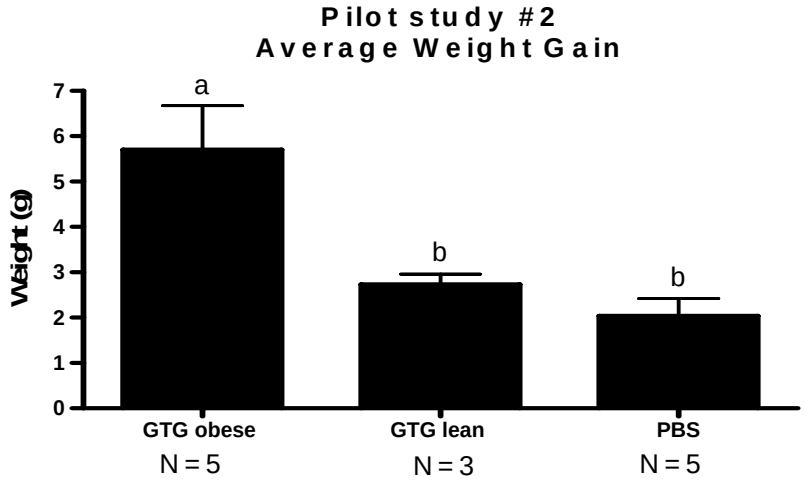

Figure 2: 10-week weight gain during pilot study 2. ANOVA P = 0.0077; GTG obese versus GTG non-obese P < 0.05; GTG obese versus PBS P < 0.01; GTG lean versus PBS P > 0.05.

TRAMP MICE

26-week cohort

Based on results from the pilot study we decided to use the higher GTG dose to obtain as many obese mice as possible. The 26-week cohort was started first and 33 male TRAMP mice between 25 and 27 weeks of age were injected with a GTG dose of 0.8 g/kg body weight. A control group was made up of 12 male TRAMP mice that were injected with the same dose of PBS. However, despite our success in the pilot study nineteen mice became ill after receiving GTG and were euthanized. Of the remaining mice, fifty-seven percent (8 out of 14) became obese. The growth curve for these mice is shown in Figure 3.

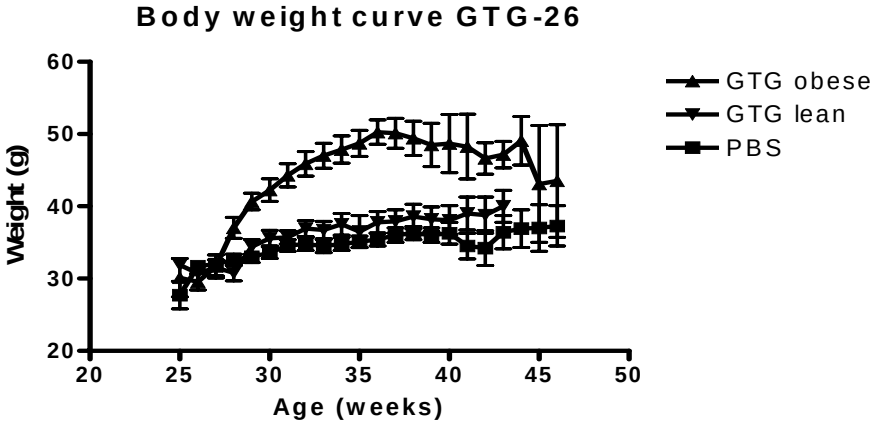

Figure 3. Body weight curve for mice in the 26-week cohort. GTG obese (▲) N=2-8, depending on age; GTG lean (▼) N=1-6, depending on age; PBS (■) N=2-12, depending on age. ANOVA P < 0.0001; GTG obese versus GTG lean P < 0.001, GTG obese versus PBS P < 0.001, GTG lean versus PBS P > 0.05.

As shown in Figure 4, the GTG obese mice gained significantly more weight than either the GTG non-obese or the control mice. There was no significant difference in the weight gain between the non-obese and the PBS-injected mice.

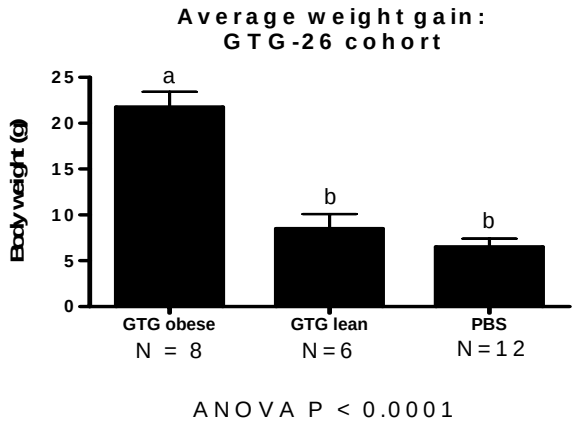

Figure 4. Weight gain of the GTG-26 cohort. ANOVA P < 0.0001; GTG obese versus GTG lean P < 0.001; GTG obese versus PBS P < 0.001; GTG lean versus PBS P > 0.05.

All mice in this cohort have reached terminal age or been euthanized for tumor burden. As shown in table 1 wAt euthanization, GTG obese mice had significantly higher final body weight and fat pad weight when compared to either GTG lean or PBS injected mice. Age at death, age at tumor palpation and urogenital (GU) tract weights were not different among the groups. We have not received final pathology reports yet but based on earlier studies we do not anticipate that tumor grade will be affected.

Table 1. End point comparison of GTG-26 mice

	Final body weight (g)	Age at death (weeks)	Age at tumor palpation (weeks)	GU tract weight (g)	Fat pad weight (g)
GTG obese (N=8)	48.6 ± 2.6^{a}	42.1 ± 1.2^{a}	33.4 ± 0.9^{a}	$^{*}9.1 \pm 1.5^{a}$	$^{*}2.6 \pm 0.3^{a}$
GTG lean (N=6)	39.1 ± 2.5^{b}	43.0 ± 1.7^{a}	33.5 ± 1.1^{a}	$^{\#}8.9 \pm 1.5^{a}$	$^{\#}1.1 \pm 0.3^{b}$
PBS (N=12)	36.7 ± 1.63^{b}	41.3 ± 1.4^{a}	32.2 ± 1.1^{a}	$^{\$}8.1 \pm 1.0^{a}$	$^{\&}0.7 \pm 0.1^{b}$

Columns with different superscripts are significantly different at P < 0.05
*N=6: two mice had post-mortem autopsies; therefore no organ weights were available
$^{\#}$N=5: one mouse had a post-mortem autopsy; therefore no organ weights were available
$^{\$}$N=11: one mouse had a post-mortem autopsy; therefore no organ weights were available
$^{\&}$N=10: one mouse had no visible fat pads

16-week cohort

Once the 26-week cohort was filled the 16-week group was started. GTG was injected into 15 male TRAMP mice at a dose of 0.8 g/kg body weight. A control group was made up of seven male TRAMP mice that were injected with the same dose of PBS. All mice were injected at 16 weeks of age. Thirteen mice became sickly after receiving GTG and euthanization was necessary; one mouse receiving PBS was euthanized because his health deteriorated following injection. Due to the high mortality following GTG injection, a lower dose of 0.5 g/kg body weight was used. Twenty-three were injected with GTG at this dose and seven with PBS, all at 16 weeks of age. Twenty-one of those receiving GTG became sickly following their injections and euthanization was necessary; none were euthanized after PBS injection. One GTG mouse survived four weeks following injection, but never gained weight from his baseline value, so the GTG group includes only three

animals. One mouse (33%) became obese. Seven mice are currently being followed in this cohort in age from 32-40 weeks of age. Body weight curves for these mice are shown in Figure 5.

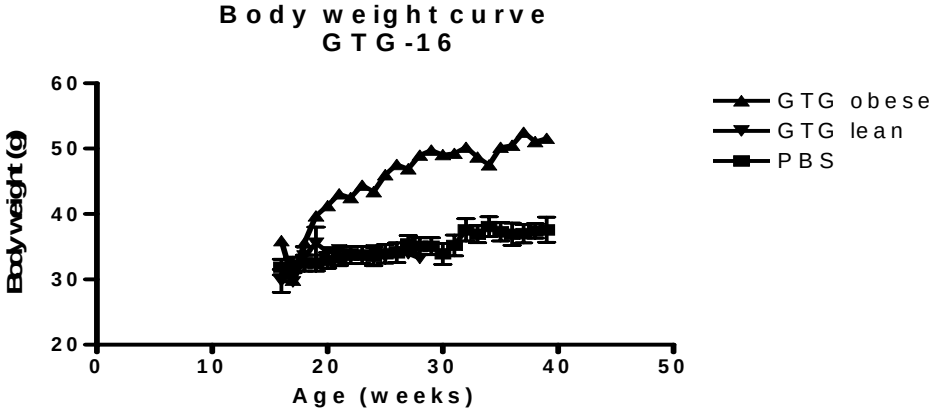

Figure 5. Body weight curve for mice in the 16-week cohort. GTG obese (▲) N=1; GTG lean (▼) N=2; PBS (■) N=2-13, depending on age. ANOVA P < 0.0001; GTG obese versus GTG lean P < 0.001, GTG obese versus PBS P < 0.001, GTG lean versus PBS P > 0.05.

6-week cohort

As it became apparent that the dose of GTG was resulting in high mortality it was lowered to 0.5 g/kg body weight. Thirty-one TRAMP mice were injected with GTG at this dose and ten with PBS, all at 6 weeks of age. Twenty-four of those receiving GTG became ill following their injections and had to be euthanized; none were euthanized after PBS injection. Seventy-one percent (5 out of 7) are obese. Nineteen mice remain in the study, ranging in age from 22-27 weeks of age. Body weight curves for the 6-week cohort are shown in Figure 6.

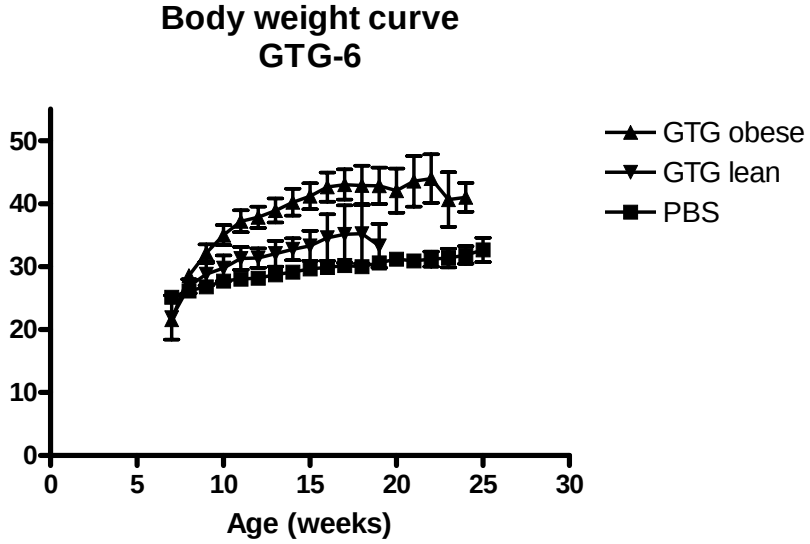

Figure 6. Body weight curve for mice in the 6-week cohort. GTG obese (▲) N=2-5, depending on age; GTG lean (▼) N=2; PBS (■) N=5-15, depending on age. ANOVA P < 0.0001; GTG obese versus GTG lean P < 0.001, GTG obese versus PBS P < 0.001, GTG lean versus PBS P > 0.05.

In a final attempt to use the GTG approach we altered the injection protocol to include two injections, 48-hours apart, each at 0.4 g/kg body weight. We hoped this lower dose would be better tolerated in the 6-week old mice. However, we still a high percentage of mortality, so this protocol is no longer being used.

Future Direction

To say the least this has been a very frustrating experience. We had every reason to believe based on the literature and our preliminary data in male mice that use of GTG would provide a straight-forward approach to induce obesity at specific ages. We have contacted people who have recently published studies using GTG and when speaking with them they do acknowledge toxicity as a problem but this has not been addressed in the manuscripts and they have offered little useful information.

We have therefore decided to take a somewhat different approach using a prostate cancer cell line developed from the TRAMP mice [25]. We will use wild type C57BL6 male mice, the strain on which the TRAMP mice are maintained, and will inoculate them with the cell line. This approach has been used in several recently published studies [26;27]. C57BL6 male mice (n=160) will be obtained from breeding colony maintained at the Hormel Institute or will be purchased from Jackson Laboratory, Bar Harbor ME. From weaning at 4 weeks of age mice will be maintained on AIN-93M diet (Table 2). At 6 weeks of age 120 mice will be switched to the AIN-93M-High-Fat diet (Table 2). When C57BL6 mice are fed high-fat diets Obesity-Prone and Obesity-Resistant groups can be identified [28]. Serum samples will be obtained at 26 weeks of age to determine serum leptin levels and mice will be stratified by body weight classification based on weight gain from 6-26 weeks of age. Based on three previous trials approximately 2/3 of the mice gain weight (2 standard deviations above controls) and are classified as Obesity-Prone and/or Overweight. The remaining high-fat diet mice are designated as Obesity-Resistant as they remain in the body weight range of the low fat (AIN-93M) diet mice. At this time the 40 heaviest mice will be assigned to Obesity-Prone group, the middle weight will be assigned to Overweight and the lightest third to the Obesity-Resistant group. The middle third will be designated as Overweight. At this time one half of the mice in each group will be switched to the low-fat diet for the remainder of the experiment. Additionally at 26 weeks of the 40 mice maintained on the AIN-93M diet half will be switched to the high-fat diet to determine the effect of the diet per se on tumor development.

Table 2. Composition of Experimental Diets

	AIN-93M[1] (g/kg)	AIN-93M-High-Fat[2] (g/kg)
Casein	140.0	190
L-Cystine	1.8	2.44
Corn starch	470.692	305.95
Maltodextrin	160.0	104
Sucrose	100.0	65
Soybean oil[3]	40.0	160
Cellulose	40.0	98.4742
AIN-93-MX -Mineral mix	35.0	47.25
AIN-93-VX- Vitamin mix	10.0	13.5
Choline bitartrate	2.5	3.375
TBHQ (antioxidant)	0.008	0.0108

[1]Based on AIN-93M diet designed for long-term maintenance of rodents. [2]Updated high fat diet based AIN-93M diet.
[3]Although soybean products such as soy meal and protein are associated with anticarcinogenesis, this is not true for soybean oil. Soybean oil improves the fatty acid n-6/n-3 ratio. Corn oil used in AIN-76A diet with high n-6 fatty acid content was thought to support tumor growth. No phytoestrogens have been found in AIN-93 diet.

At 26 weeks of age at the time of obtaining blood samples all mice will be subcutaneously inoculated with TRAMP-C1 prostate cancer cell lines ($2x10^6$ cells) as a 200 μL suspension in 50% Matrigel. Cells will be

grown and will be maintained in RPMI 1640 medium containing fetal bovine serum supplemented with penicillin (100 units/mL) and streptomycin (100 μL). Following cell inoculation mice will be followed for an 8 additional weeks to determine age of tumor detection (latency), incidence and rate of tumor growth. In order to Body weights will be assessed weekly and mice examined for tumor development. The age of tumor detection will be based on the ability to measure a growth of 5mm. Once tumors are identified they will be monitored twice weekly to ensure health status of the mice. Tumor length (l) and width (w) will be assessed with calipers, and tumor volume calculated ($V = 0.4 \times l \times w^2$). Mice will be euthanized for the following reasons; tumors reach 20 mm in diameter, weight loss of 20% occurs, tumors ulcerate, there are other health related issues or the mouse reaches the terminal age of 34 weeks. Three hours prior to sacrifice mice from each group will be injected with 5-bromo-2'deoxyuridine (BrdU) for determination of tumor apoptosis and proliferation rates. At euthanasia, blood will be obtained for serum leptin and adiponectin determinations. Tumor samples will be prepared for histopathological analyses, as well lymph, lungs, livers and spleens to determine metastasis rates. Urogenital tracts will be removed and weighed. Tumor weight relative to body/carcass weight will be calculated to assess tumor burden. Retroperitoneal and epididymal fat pads will be dissected and weighed and used as a surrogate of body fatness.

We have obtained the TRAMP-C1 cell line and have submitted a change of protocol to our IACUC to undertake this study. The results will indicate how body weight and serum factors associated with obesity impact tumor development.

KEY RESEARCH ACCOMPLISHMENTS:

1) Attempted to undertake experiments as described to induce obesity at specific ages.
2) Found that toxicity and mortality associated with GTG-induced obesity makes it not practical for continued use.
3) Beginning to establish an alternative approach to address the issue of the effect of body weight on prostate cancer development.

REPORTABLE OUTCOMES:

None to date but we are sending an abstract for the September DOD prostate cancer meeting.

CONCLUSIONS:

As indicated this has been a frustrating experience over the past year trying to undertake what we thought was a straightforward approach to inducing obesity in the mice. At this time we hope that our new approach will provide more insightful results into the effect of obesity on prostate cancer.

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